Award Number: **W81XWH-12-1-0591**

TITLE: **Organizing the Cellular and Molecular Heterogeneity in High-Grade Serous Ovarian Cancer by Mass Cytometry**

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REPORT DATE: **October 2016**

TYPE OF REPORT: **Annual**

PREPARED FOR: **U.S. Army Medical Research and Materiel** Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual	30 Sep 2015 – 29 Sep 2016

4. TITLE AND SUBTITLE
Organizing the Cellular and Molecular Heterogeneity in High-Grade Serous Ovarian Cancer by Mass Cytometry

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W 81XW H-12-1-0591

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Stanford Junior University
Stanford, CA 94305-2004

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Tumor heterogeneity in high grade serous ovarian cancer (HG-SOC) represents a significant barrier for successful therapeutic intervention. To further understand the cell types contributing to this heterogeneity we performed a comprehensive phenotypic characterization of 22 primary ovarian tumor samples. Our unsupervised analysis revealed shared and circumscribed patterns of tumor cell types across multiple HG-SOC primary samples. In addition to identifying cells characteristic of epithelial tumors we found several repeatedly observed, though previously unrecognized, cell types. These included three unique E-cadherin-expressing cell subsets, cell subsets co-expressing E-cadherin and vimentin and critically one subset that co-expressed high levels of all stem cell markers interrogated. Poorer prognosis tumors had an increased frequency of cells co-expressing vimentin, HE4 and cMyc and also showed greater overall phenotypic heterogeneity quantified by Simpson's Diversity Index. Importantly the novel cell types identified have the potential to become a focus for developing new therapies as well as a means of monitoring the disease.

15. SUBJECT TERMS
Tumor initiating cells, Modularity Optimization in Networks of Cellular Phenotypes (MONOCLE), primary diagnostic samples, Indivumed, dissociation conditions, evaluation of viability and apoptosis, immunohistochemistry, validation of tumor antibodies, validation of immune cell antibodies, ovarian cancer cell lines, future plans.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	21	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

High-grade serous ovarian cancer (HG-SOC) is the fifth most lethal cancer in women and the most lethal of gynecological malignancies[1,2]. Most often diagnosed at more advanced stages, a great challenge in treating HG-SOC is the apparent large number of disease subclasses based on genetic analyses[1,3-6]. Defective DNA repair mechanisms are characteristic of the disease and are most likely responsible for the extensive genetic abnormalities, most frequent of which are focal copy number alterations and epigenetic modifications, confounding a systematic approach to successful treatment the disease. Furthermore, given the genetic plasticity of HG-SOC each patient can manifest one disease at diagnosis and other subtypes over time. At present, platinum-based therapeutic regimens are the most commonly used in the clinical settings of first diagnosis and post-relapse. Frequently a more aggressive platinum resistant form emerges. According to a seminal review by Vogelstein et al. the vast array of genetic events found in cancer all converge on three essential cellular processes, cell fate, cell survival and genome maintenance all regulated by twelve intracellular signaling pathways[7]. This is consistent with cancer having a "structure".

The hypothesis of our DoD proposal is that in spite of the vast range of genetic aberrations detected in HG-SOC, there must exist a unifying architecture that links biology to pathology across these tumors. By dissecting diagnostic, chemo-naïve clinically annotated HG-SOC primary samples into single cells for analysis of their phenotypes and signaling states, at the *deepest possible resolution currently available*, we will provide a unifying vision of *ovarian cancer "systems biology"* to bring about more informed changes to treatment modalities. To accomplish this vision with HG-SOC, we are using a single cell technology, mass cytometry, or CyTOF (**Cy**tometry by **T**ime-**O**f-**F**light), largely developed in our laboratory, for immunologic and cancer cell studies[8-12]. CyTOF uses antibodies conjugated to chelated metal ion tags, allowing for the simultaneous measurement of up to 40 parameters on a cell-by-cell basis, including surface markers and intracellular signaling proteins. CyTOF has been applied to complex tissues such as blood, bone marrow and, recently, ovarian ascites as well as single-cell suspensions derived from primary HG-SOC tumors.

Over the past year, we have greatly expanded the scope of our analysis and revealed new tumor cell types associated with the biology and clinical course of HG-SOC. Furthermore, our analysis of the immune cell infiltrate has confirmed our earlier findings where we discovered a decidual-like NK cell subset that correlates with tumor abundance. We have performed functional co-culture experiments with an NK cell line and HG-SOC tumor cells to understand the interplay between NK cells and tumor cells. This year the focus was on developing new analysis approaches, one of which was to perform correlation analyses between tumor cell subsets, immune cell subsets and tumor and immune cell subsets. This data will be presented in this report. ***One of our most important findings from the study of 22 primary ovarian tumors was that the heterogeneity of HG-SOC was far more circumscribed than might have been predicted based on genetics***. ***We have identified three new tumor cell types that co-occur across tumors. In addition we have identified new therapeutic target requiring further study with larger tumor sample cohorts that could benefit HG-SOC patients. The results from our study on the tumor cell compartment are for imminent submission for publication.***

Text in green font is the update of aims for this year's report with updates from our 2014 report (red font) and from our 2015 report (blue font) left for comparison. Within the body of the text, a detailed discussion will be provided for our progress over the last year, which continues to build on **Task1** as well as report on studies initiated for **Task 2** and some exciting new findings in our tumor immune studies in **Task 3.**

KEYWORDS
Serous ovarian cancer, primary tumors, mass cytometry, single cell, antibodies, stem cells, heterogeneity, epithelial mesenchymal transition, relapse, Simpson's Index of diversity, immune compartment, clustering, correlation analyses, decidual-like NK cells, NK cell ligands, T cells, macrophages, immune checkpoints.

OVERALL PROJECT SUMMARY

A. Background

Single mass cytometry facilitates high-dimensional, quantitative analysis of the effects of bioactive molecules on cell populations at single-cell resolution[8-12]. Datasets are generated with panels of 41 optimized antibodies, in which each antibody is conjugated to a polymer chelated with a stable metal isotope, usually in the Lanthanide series of the Periodic Table[9,11,13]. The antibodies recognize surface markers to delineate cell types, such as immune, epithelial, mesenchymal, and intracellular signaling molecules demarcating multiple cell functions such as survival, DNA damage, cell cycle and apoptosis. By measuring all these parameters simultaneously, the signaling network state of an individual cell can be measured. The ultimate goal of this work, and beyond, will be to assign molecular status and function to cell subsets defined by 40 parameters at the single cell level.

B. Overview of status of tasks

Task 1

Subtask 1a. Establish conditions for dissociation of solid tumors into single cells that maintain cells' ability for functional signaling. **Done with protocols transferred to Indivumed Inc, Hamburg Germany and now routine. We continue to procure de-ideintified samples from Indivumed who are now the preferred provider of quality samples to the NCI.**

Subtask 1b. Select a panel of extracellular modulators with which to measure signaling responses in both tumor cells and peripheral blood cells. **A preliminary list of modulators has been made including but not limited to, TBFβ, BMP2, EGF, TGFα, heregulin, amphiregulinn, LPA, IL6, LPS, IL6, IFNα, and IFNγ has been made and protocols for exposing single cell dissociation of primary tumors are in the process of being transferred to Indivumed. Work in progress is prioritizing this list. This next phase will involve transferring our protocols to Indivumed which is planned for later in the year. We are currently performing studies with HG-SOC cell lines treated with TBFβ as a means to monitor EMT and recapitulate the findings of the cell types we identified in primary tumors that co-express E-cadherin and vimentin. This will permit us to design in vitro models and iterate back to tumors.**

Subtask 1c. Select two panels of ~40 antibodies each. Done. **We constructed two antibody panels in which the second was a variant of the first based on a mass cytometry experiment with six primary samples. *The data from two independent experiments with each panel will be described in the body of the text.* We assembled three panels with 40 antibodies each. One panel was focused on the tumor cells and the two others on the tumor-immune infiltrate (Table 1)**

Subtask 1d. We have submitted the necessary HRPO (IRB) and the ACURO and are awaiting approval. **Done**

Subtask 1e. Acquire 10 primary diagnostic (no treatment) ovarian tumor or ascites samples with matched blood samples. **Done. We have performed two mass cytometry experiments: i) six primary naïve tumors and ten HG-SOC ovarian cell lines described to be genetically most similar to primary HG-SOC[14]. We acquired 22**

samples which were all processed for CyTOF with the three antibody panels.

Subtask 1f. Develop and apply new informatics tools and algorithms to the data generated from subtask 1d (Nolan lab and Pe'er lab at Columbia) (these efforts will be ongoing throughout mos of the duration of this award) **New tools developed: from the Nolan Lab: Citrus[15], X-shift[16], Gatefinder (unpublished), Pe'er Lab: DREMI[17]. A manuscript describing X-shift has been submitted and a manuscript about gatefinder is accepted for publication in nature Biotechnology. We are using these algorithms in combination with standard statistical tools for analyzing our data. The X-shift clustering algorithm specifically developed for CyTOF datasets was published earlier this year in Nature Methods[16].**

Subtask 1g. Pending data from subtask 1e modify antibody panels. Titrate any new antibodies (3-36 months. Anticipate continuous low-level activity for this subtask throughout the award period). ***See subtask 1c.*** **Our three new and modified antibody panels include new antibodies which were all conjugated and titrated. Concentrations where signal to noise was maximal were selected for our CyTOF experiments. The antibody panels were finalized last year and activities surrounding panels were replenshments.**

Subtask 1h. Acquire >er than 150 primary diagnostic (Neel lab at UHN Toronto, and Berek at Stanford) serous ovarian cancer samples (from Neel at UHN and Berek at Stanford) and process for mass cytometry with modified panels (6-40 months). Twenty five of these will be processed for xenotransplant (the Neel Lab currently has Research Ethics Board approval to conduct all of the tests described), requiring 10 mice for each subject tumor for 250 mice. **In progress. Continues in progress. Continues.**

Subtask 1i. Using SPADE and other algorithms, segregate and aggregate cell subsets in hierarchical pattern with intracellular and cell surface marker combinations. **Using a new deterministic K-nearest neighbor-clustering algorithm, we see important relationships between tumor cell subsets. This information will be presented in the body of the text. We analyzed the new set of 22 HG-SOC samples with this algorithm and noted some new findings especially as they pertain to the tumor and immune cell compartments. This analysis will be presented. The new analysis from of the last reporting period identified key attributes of HG-SOC tumors and this work will be presented.**

Subtask 1j. Building of subset space in relationship to therapy/outcome (6-48 months). **We have not run enough samples and also for those we have run, not enough time has elapsed to fully evaluate patient outcome. We are expecting the outcomes data regarding platinum sensitivity in the next couple of weeks and may not be able to incorporate those findings into this report. We have identified a tumor cell subset that pre-exists in diagnostic samples that is predicts relapse. Additionally we have quantified heterogeneity using the Simpson's index of diversity that considers HG-SOC tumors as an ecosystem.**

Subtask 1k. Assess relative tumor-initiating properties of cell subsets from subtask 1h with established quantitative xenograft assay (Neel lab, 6-40 months). **In progress. In the next year we plan to FACS-sort the three new tumor cell subsets we identified and perform functional studies such as growth in soft agar combined with patient-derived xenograft models.**

Task 2

Previous work from the Nolan group showed that measuring the signaling responses of cancer cells to perturbations is more informative than assessing basal phosphorylation states. This task is focused on measuring signaling responses to extracellular perturbants such as growth factors, cytokines and drugs with relevance to ovarian cancer. In this task, the objective will be to uncover druggable pathways in serous ovarian cell subsets within and across primary samples.

Task 2 has subtasks that are dependent and independent of Task 1. For Task 2 we have set up foundational studies to measure drug responses in HG-SOC cell lines. Specifically, we have set MTT assays (colorimetric readout) to measure the effects of drugs on proliferation, and growth in soft agar assays. We are evaluating carboplatin and paclitaxel and other investigational agents such as PARP inhibitors, JQ1 (an epigenetic modifier) and others that are under evaluation based on our primary tumor work in Task 1. Due to the relative immaturity of these studies, we will focus this report on the 1c, g and i. **We have established growth in soft agar assays using HG- SOC cell lines for evaluating their tumorigenic potential with and without drugs. In the past few months we have focused on PARP inhibitors. The assays is ready for application to cell subsets isolated from primary tumors which is imminent. Over the last year we have directed our drug studies around two classes of targeted inhibitors, the data of which will be presented. The first is the screening of HG-SOC cell lines with bromo-domain inhibitors based on our CyTOF analysis. The second class is the PARP inhibitors, in recognition of their surge into the clinic for HG-SOC patients.**

Task 3

Although the presence of infiltrating cytotoxic T cells correlates with good prognosis, whereas regulatory T cells correlate with poor prognosis in SOC, there is limited understanding of the factors that contribute to the generation of these opposing responses. Understanding the mechanisms by which a given tumor microenvironment is able to promote immune surveillance could eventually lead to the clinical development of biomarkers that could select patients responsive to immune therapy. We will use mass cytometry to evaluate the tumor microenvironment in the same SOC samples as above utilizing antibodies against immune cell subsets. **We analyzed 22 HG-SOC primary samples from Indivumed with two panel focused on the immune infiltrate. The panels included checkpoint inhibitors and the data will be presented in detail. From our continued analysis we identified a subset of NK cells that correlated with tumor cell abundance. We provide our detailed analysis regarding the interplay between NK cells and specific tumor cell subsets that importantly identify potential new targets for HG-SOC.**

Subtask 3a. Assemble panel of extracellular modulators based on the known biology of the cell types that infiltrate ovarian tumors; immune cells, endothelial cells and stromal cells. **Ongoing *Subtask 3b***. Validate reagents to monitor signaling pathways mediated by extracellular modulators in cell lines and peripheral blood. (1-24 months). **We have available a large repository of agents (growth factors, cytokines and drugs) with which to characterize immune cell subsets from peripheral blood taken from HG-SOC patients. We are currently prioritizing which agents to use. Ongoing. See Task 2**

Subtask 3c. Acquire 10 primary serous ovarian cancer samples with which to test response of tumor infiltrating cells to extracellular modulators identified in 3a. **(Ongoing). Ongoing. Ongoing.**

Subtask 3d. Culture tumor-infiltrating lymphocytes from samples in Subtask 3c and characterize them for cytokine and chemokine production. (Ohashi lab 12-24 months). **This subtask has changed and the Nolan Lab is generating enriched immune fractions from primary tumors and establishing in vitro assays to determine immune-suppressive versus immune-enhancive activities of the tumor immune compartment. Based on our data with the tumor immune cell infiltrate (discussed in the body of the text) we are following up with our findings regarding NK cell subsets and performing co-culture experiments between peripheral NK cells and HG-SOC cell lines which are discussed in the text below. Data will be presented.**

Subtask 3e: Acquire >er than 150 primary serous ovarian cancer samples (Neel lab at UHN Toronto, and Berek at Stanford) with which to test response of tumor infiltrating cells to extracellular

modulators identified in 3a. (24-50 months). **In progress.**

Subtask 3f*.* Culture tumor-infiltrating lymphocytes from samples in Subtask 3c and characterize them for cytokine and chemokine production. **See Subtask 3d.** ***Subtask 3g****:* Using SPADE and other algorithms, segregate and aggregate tumor infiltrating cell subsets in hierarchical pattern with intracellular and cell surface marker combinations. Build computational models that correlate intracellular signaling responses in tumor infiltrating cell subsets with intracellular signaling responses of tumor cells with clinical outcomes (12-60 months). **In progress and update will be in body of text. This is a continuous activity for us and the latest data will be discussed in the text below. New data analysis will be presented.**

C. Description of studies and results

The bulk of this report will describe the continued data analysis of 22 primary OC tumor samples with over 100 antibodies providing, to date, the most comprehensive single cell proteomic analysis of HG-SOC that examines both the tumor and immune cell compartments. One major result to emerge is that the heterogeneity of HG-SOC is far more circumscribed than would be predicted. Application of single cell CyTOF has identified three new cell types that define this more limited heterogeneity and of greatest relevance to patients we identify potential new therapeutic targets.

C.1 Introduction and Background

As in years 1, 2 and 3, we continue to pay close attention to obtaining samples of the highest quality, minimizing their ischemic time and optimizing all pre-analytical variables. With Indivumed Inc. in Hamburg we have highly stringent protocols in place that are now routine. All the primary samples that we evaluated were processed within 4 hours including transit time. We continue to emphasize these critical *initial steps which —though tedious—are critical to "trusting" the data from such precious samples as those obtained from patients with fatal diseases.* Furthermore, we have added more controls and verified that the data we generated is of the highest quality possible.

During Year 4 our data analysis continued with the development of new algorithms revealing new insights about HG-SOC heterogeneity, new tumor and immune cells types as well as correlations between tumor cells and immune cells. It is readily apparent that the depth of data generated by CyTOF requires significant mining and is revealing unrecognized and revolutionary insights into the disease. Several key results have great potential to benefit patients. Two papers are in preparation, one of which is very close to submission (November 2016).

C.2 Results

Assay performance

Given the potential of our data to be developed into clinical tests we focused further on the data quality and reproducibility. High reproducibility of our methodology was demonstrated by analysis of data from a) multiple replicates across fluorometric and mass cytometry platforms b) technical and biological replicates from two mass cytometry runs separated by a period of two weeks c) two mass cytometry runs of 11 tumor samples included in this study and separated by a period of year. Furthermore, the results reported in this study are not attributable to batch variation. A subset of the data showing remarkable reproducibility of samples analysed one year apart is shown in **Figure 1**.

Figure 1: Reproducibility demonstrated by technical replicates assayed over 1 year apart. a) Overlay depicting 2D flow plots of E-cadherin and vimentin generated from Experiment 1 (2015) and Experiment 2 (2016). b) Overlay of histograms depicting median protein expression of a selection of proteins generated from Experiment 1 (2015) and Experiment 2 (2016).

Confirmation of three new tumor cell subsets identified by CyTOF

Some of the data regarding ne cell types was presented last year but we have refined our interpretations based on further analysis and this is described below.

Primary chemotherapy-naïve ovarian tumour samples, with a median follow-up post-surgery of 28.5 months and characterized by genomic sequence analysis for *BRCA1, BRCA2* and *TP53*, were subjected to stringent protocols optimized to generate viable single cell suspensions after surgical resection. Tumour sections were analysed by immunohistochemistry (IHC) to allow subsequent comparison with single cell data.

Single cell suspensions of tumour cells were analysed by mass cytometry which detects bound antibodies via metal isotopes [11,12]. A panel of 41 antibodies, designed to interrogate key features of HG-SOC tumour-cell biology [1,18-24], was utilized. As reported last year the data were analysed and visualized by the X-shift clustering algorithm[16] (new publication) and minimum spanning tree visualization tool. We identified tumor cells that were mutually exclusive for their expression of E-cadherin and vimentin consistent with established information about epithelial tumor biology. Additionally we identified tumor cell subsets that co-expressed vimentin and E-cadherin some of which appeared to form a "bridge" in the map wherein E-cadherin levels declined concomitantly with increased vimentin expression (**Figure 2**, encircled and numbered). Furthermore, SNAIL a master coordinator of EMT, was also frequently expressed in these "bridge" clusters[19,21] (**Figure 3**).

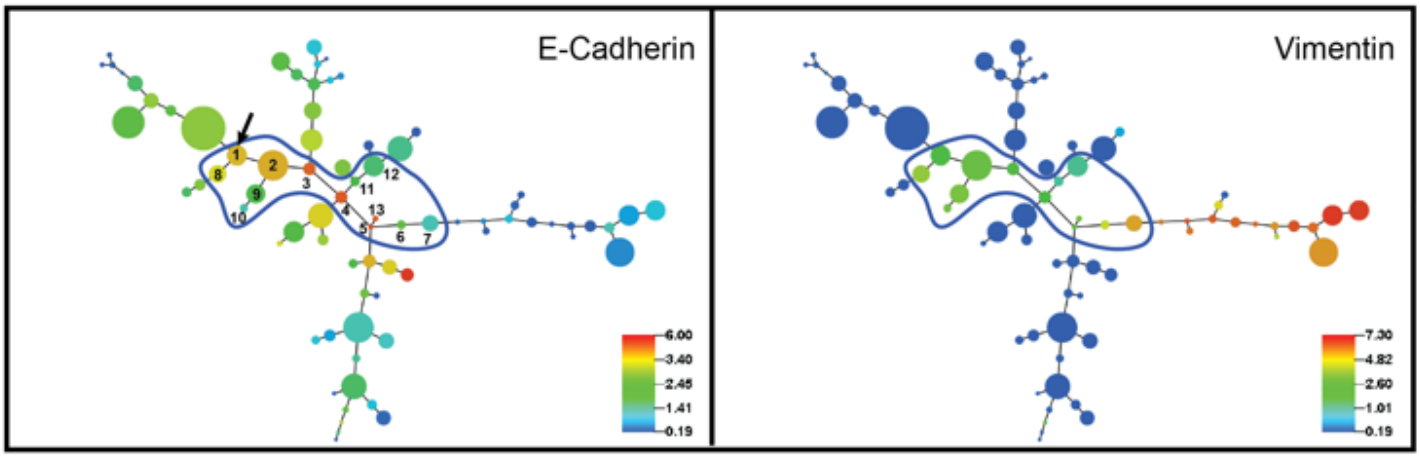

Figure 2: Identification of EV cell types (numbered and encircled) forming a "bridge" between the E-cadherin and vimentin parts of the minimum spanning tree.

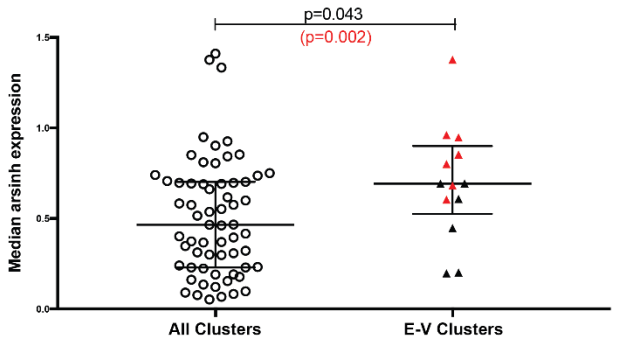

Figure 3: Arsinh-transformed median expression levels for SNAIL in the 13 EV clusters versus all other clusters in the composite MST (p = 0.043). If only the seven EV clusters (red triangles), found along the backbone of the MST (Fig. 2) are included for comparison the difference becomes more pronounced (p = 0.002).

Recent data support the link between transit through EMT and the acquisition of stem cell-like properties[21,25]. Consistent with this, the putative cancer stem cell markers included in this study (CD24, CD13, CD10, CD73, CD61, CD49f, CD90, CD44, CD133, endoglin, ROR1), as well as signalling proteins involved in conferring stemness (Sox-2, pSTAT3, pSTAT5, NFκB, pCREB and β-catenin)[25,26], were detected in the EV clusters (. All EV clusters, except for EV1 differentially expressed these putative stem cell markers. EV1 was notable for expressing, often at increased levels, all the "stemness" markers interrogated suggesting that cells in this cluster may have reached, or are transitioning through, an important checkpoint for outgrowth in the tumour compartment or are acquiring metastatic traits. In other branches of the MST, the many proteins co-expressed (in differing combinations and intensities) included stemness proteins, cell cycle proteins, DNA damage sensors and CD151, a key regulator of adhesion implicated in metastatic progression of ovarian cancer[1,23,27]. The observed phenotypic differences within the EV clusters may represent "undefined" states facilitating

adaptation to the changing dynamics of the microenvironment[28].

Examination of the MSTs on a per-sample basis showed several co-occurring features **(Figure 4**). Reflecting the observations for the composite MSTs, the majority of individual samples were enriched for cell clusters that expressed E-cadherin, rather than vimentin **(Figure 4)**.

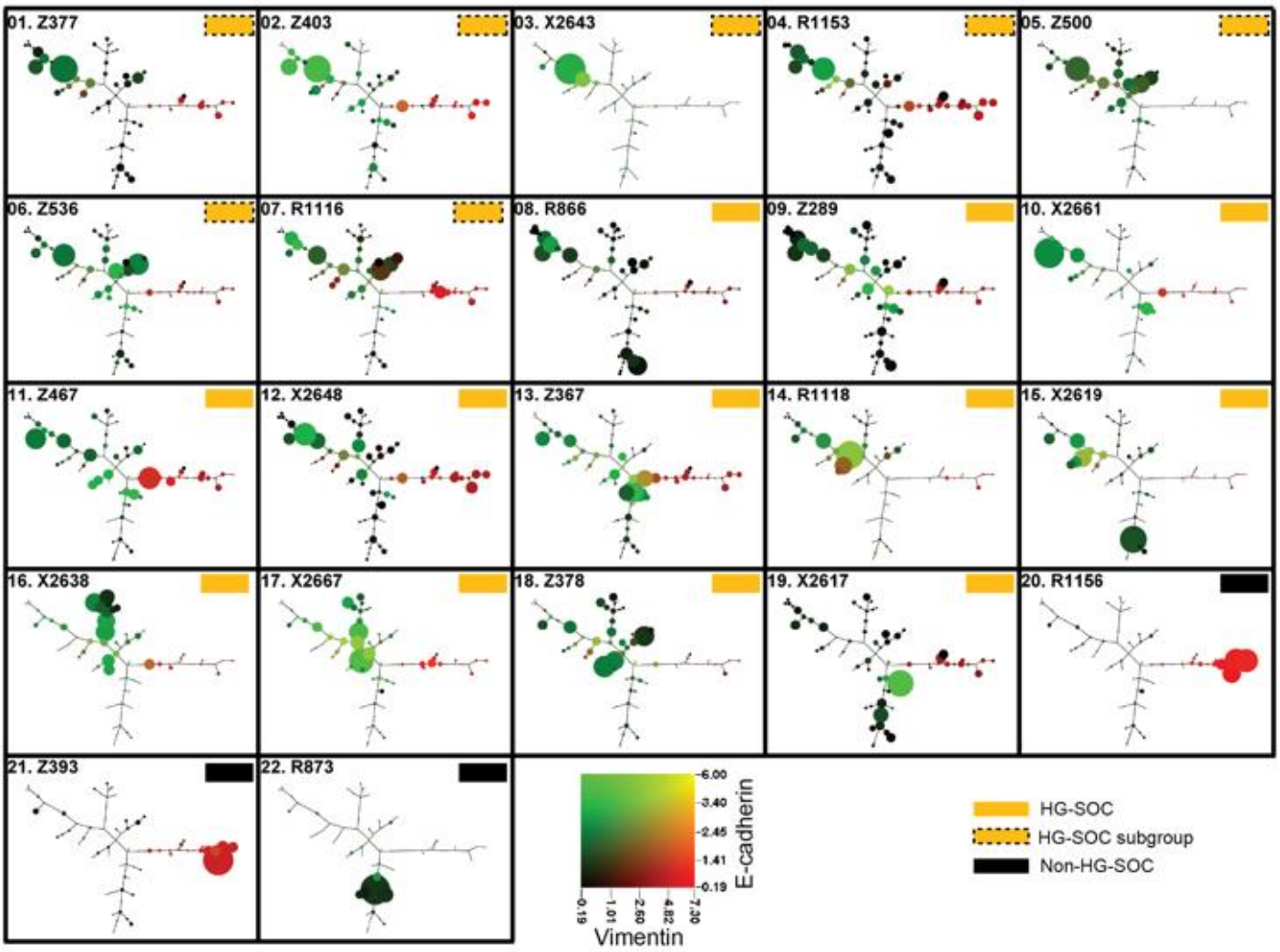

Figure 4: Individual MSTs for 22 tumor samples showing co-expression of E-cadherin (green) and vimentin (red). Bubble sizes represent the number of cells in each cluster ($\sqrt[3]{Absolute\ number\ of\ cells\ from\ all\ samples}$). HG-SOC tumor samples are denoted by an orange block in the top right-hand corner. Dotted line around the block represents a potential HG-SOC tumor sub-group. Non-HG-SOC ovarian histiotypes are denoted by a black block in the top right-hand corner of each individual histiogram.

Examination of the relative cell frequency distribution across all clusters on a per-sample basis revealed that there were three repeatedly occurring dominant clusters (termed DC 1, 2 and 3) that were located within the E-cadherin-expressing portion of the tumor **(Figure 5)**. Furthermore, for sixteen samples at least 10% of all tumor cells occupied one of these DCs and this cell frequency distribution within a DC was often much higher even reaching up to 86% of the cells within a sample **(Figure 5).**

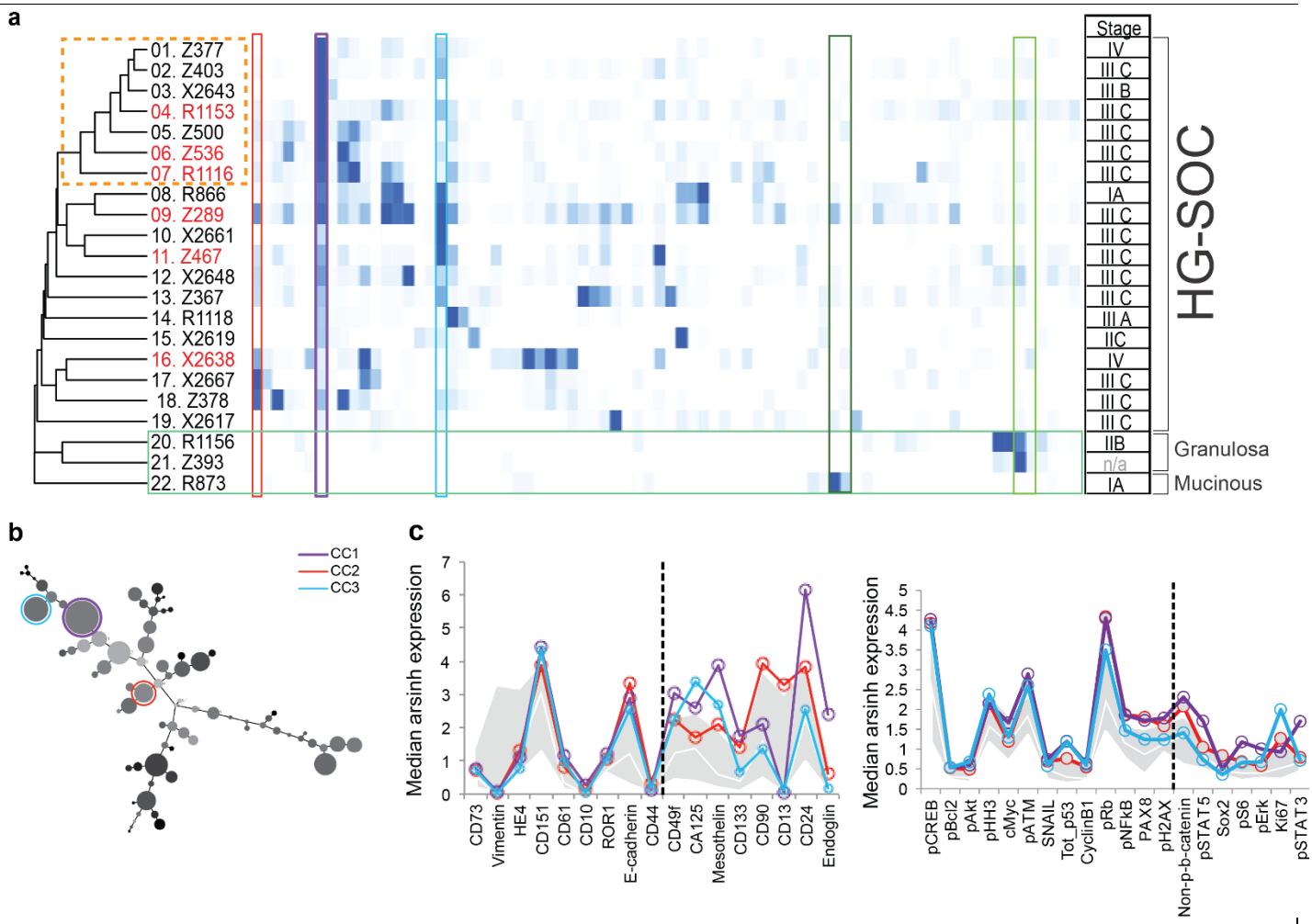

Figure 5: Novel and recurring cell subsets across individual HG-SOC tumors. a, Heat-map with dendrogram (left-hand side) depicting cell frequency distribution within clusters for individual tumors (n = 22). Three cell clusters (common clusters (CCs)) with the highest median frequency across the HG-SOC samples are depicted with vertical boxes (CC1 - purple, CC2 - red and CC3 – cyan). One potential HG-SOC subgroup is highlighted by orange box. **b**, Position of CCs on composite MST. **c**, Median protein expression in CC1, 2 and 3 (arsinh-transformed raw median counts). Surface proteins, used to cluster cells, are indicated in the left-hand plot and intracellular proteins are indicated in the right-hand plot. Vertical dashed lines set a boundary between conserved and variably expressed proteins within the three clusters. Median protein expression for all other clusters (white line) and the interquartile range (solid grey area) is shown

For samples 13, 16 and 19 the cell frequency distribution within DC1, 2 or 3 was less than 10% with alternate clusters dominating these samples suggesting that these three tumors may have a different heterogeneity structure. Three patient samples (numbered 20, 21 and 22) had MST cluster patterns that were highly divergent from the majority MSTs and notably lacked cells within the DCs. Two of these outlier samples were subsequently identified as being ovarian granulosa cell tumors, known to express high levels of vimentin[29] while tumor 22 was found to be a mucinous histiotype, with the majority of cells converging on three closely related clusters expressing low levels of E-cadherin[30]. Exclusion of the outlier histiotypes from the X-shift clustering did not appreciably change the overall structure of the composite MSTs. Overall, the data depicted by the heat-map show that for the HG-SOC samples (1 to 19) the cell frequency distribution was markedly different from the non-HG-SOC samples (20 – 22). **Moreover, hierarchical clustering based on cell frequency distributions suggested that there are recurring patterns of heterogeneity.** In particular; samples 1 to 7

appeared to be unified into a subgroup which was strengthened using bootstrap resampling. However, additional sample cohorts will be required to validate these findings and identify clinical correlates.

Protein expression profiles of DC1, DC2, and DC3 revealed 22 proteins with highly conserved expression profiles and 15 proteins with variable expression profiles (Figure. 5c, left and right of dotted line respectively). These conserved profiles may be associated with a stable or deterministic portion of the tumor transcriptome, while the variable profiles may reflect evolving stages of tumor development [31,32]. Furthermore, cell surface proteins, often used to stage tumors, showed a greater range of variability than intracellular proteins many of which are essential for both tumor survival and genomic stability [7,32]. **Additionally, compared to all other clusters identified in this study DC1 - 3, showed increased levels of pCREB, pRb, cMyc, pATM, pErk and pS6. This suggests that they might represent a common regulatory module and thus have potential as targets for therapeutic intervention.**

Identification of pre-existing cell subsets associated with relapse

All HG-SOC patients in this sample set were treated with a platinum-based therapeutic regimen after surgery [2]. However, within a year, six patients from this cohort relapsed. (**Figure 6a**, red font). Reasoning that relapse may be associated with metastases and therefore with expression of vimentin, the relapse samples contained more pre-existing cells within the vimentin clade ($p = 0.028$). Furthermore, single cell data analysis of the vimentin clade uncovered a sub-population of cells associated with relapse that co-expressed cMyc and HE4 **(Figure 6a)**. These findings were corroborated by manual-gating ($p = 0.01$ and Figure 6b) supporting the validity of the computational findings. In order to show how the presence of vimentin/cMyc/HE4 cells effected patient survival Kaplan-Meier analysis was performed. Patients' tumors were divided into two groups based on the presence of vimentin/cMyc/HE4 cells above and below a cell frequency threshold. The threshold cell frequency value was computed in two different ways. For the first, k-means clustering generated a threshold value of 3% and for the second, when samples were partitioned into equally sized groups the threshold value was 1.27%. Kaplan-Meier plots using either threshold value showed that patients with tumors having numbers of vimentin/HE4/cMyc cells greater than either threshold had a greater likelihood of proceeding to relapse ($p = 0.0005$, HR 0.17, k-means) and ($p = 0.04$, HR 0.26, equal sized groups), **(Figure 6c)**.

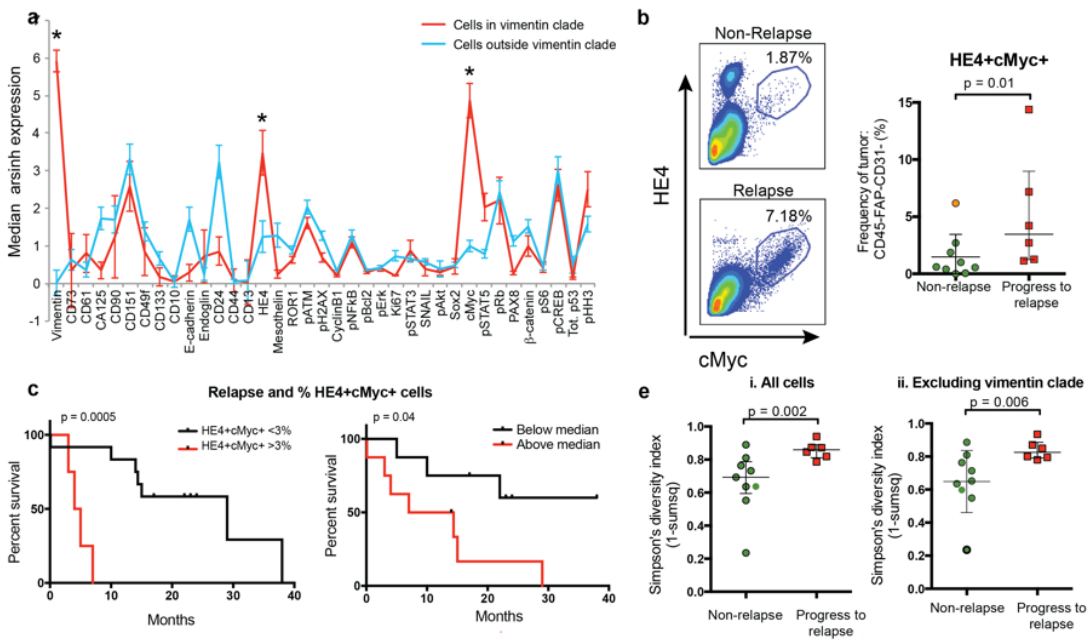

Figure 6: Novel cell types and prognosis in HG-SOC. a, Median protein expression levels (95% confidence intervals) for cells inside and outside the vimentin clade (red and blue plots respectively). Vimentin ($p = 1.62 \cdot 10^{-30}$), HE4 ($p = 0.0007$), and cMyc ($p = 2.3 \cdot 10^{-10}$) were expressed at significantly greater levels in the vimentin clade (asterisks). **b**, Manual-gating (representative samples shown) from the tumor cell parent population confirmed more cells co-expressing HE4 and cMyc in samples from patients that relapsed within one year (n = 6) versus those that did not (n = 9). **c**, Kaplan-Meier curves depict differences in time-to-relapse for samples with greater than or less than two separately derived threshold for cMyc/HE4 cells (log rank test, p = 0.0005 (k-means) and p = 0.04 (equal sized groups)).

We are actively finding partners/collaborators with large repositories of HG-SOC slides in order to initiate the development of a predictive test for relapse based on the presence of HE4/cMyc cells with the hope of predicting not only whether relapse will occur but when. We have also initiated drug screening tests using HG-SOC cell lines[14] to determine their sensitivity to bromo-domain small molecule inhibitors. These inhibitors are known to be efficacious in the setting of cMyc expression[33].

Quantification of heterogeneity by Simpson's index of diversity

To quantify the degree of heterogeneity within and between the studied HG-SOC tumors, we computed the phenotypic diversity of all the cell clusters with Simpson's Diversity Index, used recently in a study involving mass cytometry to characterize natural killer cell subtypes [34]. Here, greater heterogeneity (higher value of Simpson's Diversity Index) was observed for tumors from patients who subsequently relapsed within a year irrespective of whether vimentin-expressing cells were included (p=0.002) or excluded from the computation (p = 0.006, **Figure 6e**). This agrees with data from other studies showing that increased tumor heterogeneity is associated with poor survival [35,36]. Furthermore these data demonstrate that it is the degree of diversity rather than the proteins themselves that correlate with a poor prognosis.

Correlation analyses

The wealth of CyTOF data we obtained is amenable to correlation analyses that allows us to observe

dependencies of cell types on each other. For example are there certain cell clusters that correlate with the vimentin-expressing cell types and could therefore be associated with metastasis **(Figure 7)**?
We therefore determined the pairwise correlations for all the cell clusters and features **(Figure 7)** for all samples. The heatmap (Figure 8) shows the average spearman correlation coefficient for each pairwise correlation across the samples. Key results from this analysis: the correlation between DC1 and EV1suggest a dependency of these populations with each other. The anti-correlation between the Simpson's index of diversity with the DC1 and EV1 cell clusters suggests that these cell populations contribute to diversity.

In a separate pairwise correlation analysis performed with both tumor and immune cell subsets we demonstrated that decidual-like NK cell subsets was *positively* correlated with tumor cell abundance (presented last report and repeated with independent samples) and a non-exhausted T-cell subset was *negatively correlated* with tumor cell abundance **(Figure 9)**. Noteworthy is the multitude of studies in which d-NK cells have been shown to be important for maternal tolerance of the fetus[37]. We have been actively characterizing the mechanism by which the d-NK cell subsets confer immune tolerance to the tumor cells and have preliminary data to suggest that IL35 an anti-inflammatory cytokine[38] may be a likely new and targetable candidate. We are focusing on this aspect and hope to submit a manuscript early next year. These findings have significant implications for therapeutic intervention and provide a means of personalizing treatment regimens for HG-SOC patients.

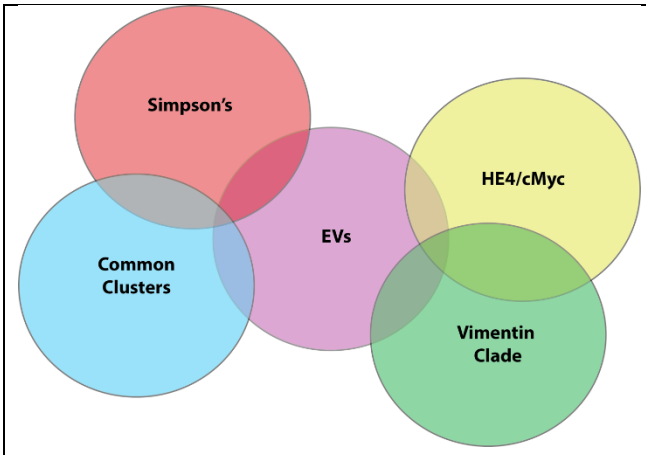

Figure 7: new cell types and features identified and examination of their correlations to each other.

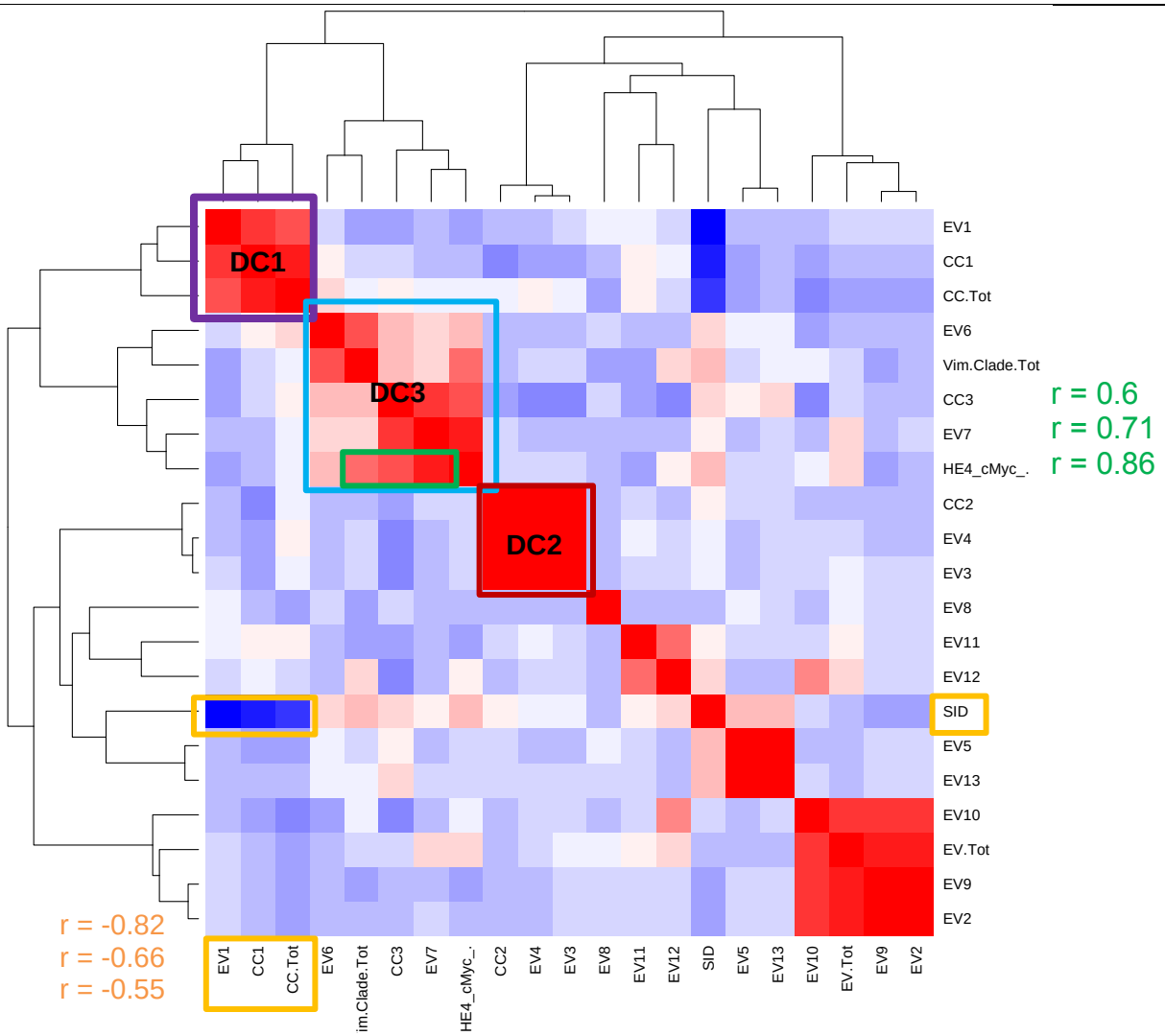

Figure 8: Correlation heatmap showing relationships between tumor cell subsets. SID Simpson's index of diversity. Correlations called out for HE4/Myc tumor cell types with vimentin clade, CC3 and EV7 (orange) and SID with EV1, CC1 and CC tot (orange).

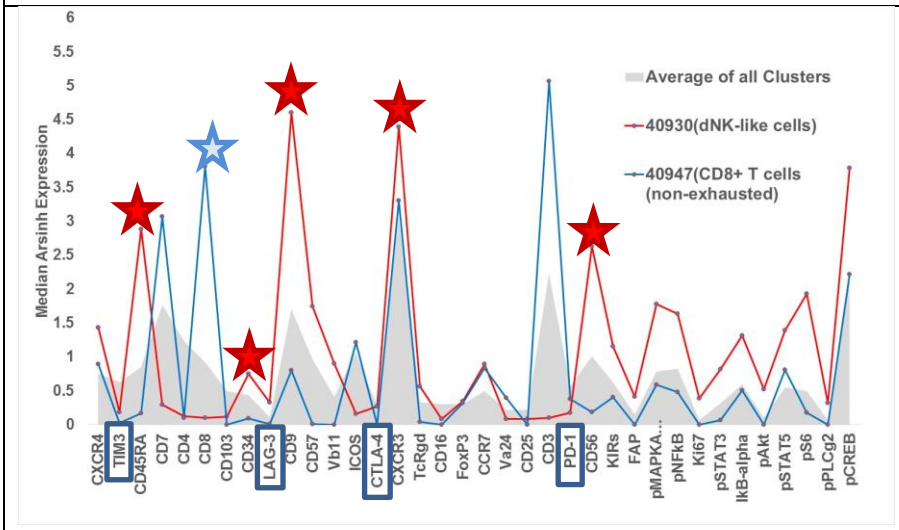

Figure 9: Protein expression in d-NK cell subset (red plot) and no-exhausted T-cell subset (blue plot). Red stars denote markers consistent with a decidual-like NK cell phenotyoe. Blue bars denote lack of exhaustion marker expression in a CD8 T cell population.

D. KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that tumor heterogeneity characterized by CyTOF is far more limited than would be expected from the genetics.
- Identified three new tumor cell subsets with potential therapeutic and prognostic benefit.
- One cell subset that co-expresses vimentin, HE4 and cMyc pre-exists in diagnostic samples and correlates with relapse. We are actively seeking partners to develop this into a diagnostic/predictive test
- More diversity within a tumor is associated with worse outcomes as quantified by Simpson's index of diversity.
- Combined our new computational tools with standard statistics; both descriptive and inferential as well as multiple hypothesis testing techniques to analyze the data.
- New correlation analyses identified new relationships between tumor and immune cell subsets.
- Specifically a decidual-like (d) NK cell subset is *positively correlated* with tumor cell abundance
- An exhausted T-cell subset is *anti-correlated* with tumor cell abundance
- The apparent immune tolerance conferred upon tumor cells by NK cells may be mediated by IL-35, thereby identifying a very new and novel target for HG-SOC tumors

E. REPORTABLE OUTCOMES

E.1 PUBLICATIONS

1. Gaudillière B, Ganio EA, Tingle M, Lancero HL, Fragiadakis GK, Baca QJ, Aghaeepour N, Wong RJ, Quaintance C, El-Sayed YY, Shaw GM, Lewis DB, Stevenson DK, **Nolan GP**, Angst MS. Implementing Mass Cytometry at the Bedside to Study the Immunological Basis of Human Diseases: Distinctive Immune Features in Patients with a History of Term or Preterm Birth. Cytometry A. 2015 Sep; 87(9):817-29.
2. Fragiadakis GK, Gaudillière B, Ganio EA, Aghaeepour N, Tingle M, **Nolan GP**, Angst MS. Patient-specific Immune States before Surgery Are Strong Correlates of Surgical Recovery. Anesthesiology. 2015 Dec; 123(6):1241-55
3. Frei AP, Bava FA, Zunder ER, Hsieh EW, Chen SY, **Nolan GP**, Gherardini PF. Highly multiplexed simultaneous detection of RNAs and proteins in single cells. Nat Methods. 2016 Mar; 13(3):269-75.
4. Spitzer MH, **Nolan GP**. Mass Cytometry: Single Cells, Many Features. Cell. 2016 May 5; 165(4):780-91. Review.
5. Samusik N, Good Z, Spitzer MH, Davis KL, **Nolan GP**. Automated mapping of phenotype space with single-cell data. Nat Methods. 2016 Jun; 13(6):493-6.
6. Anchang B, Hart TD, Bendall SC, Qiu P, Bjornson Z, Linderman M, **Nolan GP**, Plevritis SK. Visualization and cellular hierarchy inference of single-cell data using SPADE. Nat Protoc. 2016 Jul; 11(7):1264-79.
7. Fragiadakis GK, Baca QJ, Gherardini PF, Ganio EA, Gaudilliere DK, Tingle M, Lancero HL, McNeil LS, Spitzer MH, Wong RJ, Shaw GM, Darmstadt GL, Sylvester KG, Winn VD, Carvalho B, Lewis DB, Stevenson DK, **Nolan GP**, Aghaeepour N, Angst MS, Gaudilliere BL. Mapping the Fetomaternal Peripheral Immune System at Term Pregnancy. J Immunol. 2016 Oct 28 [Epub ahead of print]

E.2 PRESENTATIONS

1. Nolan: Protemomics and Personalized Medicine: Ongoing Partnership, January 25th 2016, Mountain View, CA. *"HiD Cell Barcoding & Imaging".*
2. Nolan: Big Data to Human Immune Responses, Feb 5th 2016, Irvine CA, *"A cell system based view of Immunity and Cancer".*
3. Nolan: Realize the Practical Applications of RNA-Seq, April 26th 2016, San Francisco*. "The Billion Cell Barcode Revolution".*

4. Nolan: Harvard Annual Pathology Retreat, May 6th 2016 Boston, MA. *"The Illusion of Tumor Heterogeneity-Order from Disorder".*
5. Nolan: The American Association of Immunologists, May 13th 2016, Seattle WA. *"High dimensional immune system Imaging and heterogeneity illusion".*
6. Nolan: Gladstone Institute, May 19th 2016, San Francisco. *"A Single Cell Systems - Structured View of Immunity and Cancer".*
7. Nolan: Actelion Pharmaceuticals Ltd, Allschwil, Switzerland, May 26th 2016. *"A Defined "Structure" for the Immune System That Reflects Immune Surveillance & Mechanistic Processes".*
8. Nolan: Changing Views in Cancer, Charité, Berlin. May 26th 2016. *"System-based single cell structure for cancer and immunity".*
9. Nolan: TxSACT, Houston Texas, June 13th 2016, *"From Molecular Scale to High Dimensional Imaging: Technology and Computation".*
10. Nolan: Gordon research conference, Hong Kong, China, July 13th 2016. *"Single cell deep profiling of cancer and immunity".*
11. Nolan: SATU Symposium, Taiwan, R.O.C., July 14th 2016. *"Single cell deep profiling of cancer and immunity".*
12. Nolan: Institute of Molecular Biology Academia Sinica, Sinica, Taipei. July 15th 2016. *"A Single Cell Systems-Structured View of Immunity and Cancer".*
13. Nolan: EarthRise at IONS, Petaluma CA. September 3rd 2016. *"The Illusion of Heterogeneity in Cancer".*
14. Nolan: Stanford Immunology Scientific Conference, Stanford CA. September 10th 2016. *"Organizing the Cancer/Immune System Heterogeneity Illusion with Deep Phenotypic Profiling".*
15. Nolan: Amgen, San Francisco, CA. September 15th 2016. *"Bringing Order to the Heterogeneity Illusion in Cancer".*
16. Nolan: Animal Model Development Workshop. Bethesda MD. September 22nd 2016. *"Organizing the Diversity in Blood and Tissue via High Parameter Analysis".*
17. Nolan: MedImmune, Mountain View, CA. September 29th 2016. *"The Illusion of Heterogeneity in Cancer".*

18. Fantl: Evan Goldberg Foundation for BRCA Research, Boston. February 4th 2016. *"Unravelling the heterogeneity of ovarian cancer to accelerate patient benefit".*
19. Fantl: Juno therapeutics, Seattle WA. February 12th 2016. *"Unravelling the heterogeneity of ovarian cancer to accelerate patient benefit".*
20. Fantl: ObGyn Faculty meeting, Stanford University, March 9th 2016. *"Unravelling the heterogeneity of ovarian cancer to accelerate patient benefit".*
21. Fantl: Systems Approaches to Cancer Biology, Woods Hole. April 4th 2016*"Molecular heterogeneity of ovarian cancer revealed by high-dimensional mass cytometric profiling".*
22. Fantl: Invited speaker MSKCC, NYC. July 15th 2016. *"Organizing the Heterogeneity of Ovarian Cancer Will Accelerate Patient Benefit".*
23. Fantl: Parker Institute for Cancer Immunotherapy at Stanford University Initiation Site Visit. June 30th 2016. *"Organizing the Heterogeneity of Ovarian Cancer Will Accelerate Patient Benefit".*

E.3 INVENTIONS PATENTS AND LICENSES

Non-provisional patent application:
"Methods of prognosis and diagnosis of ovarian cancer". US 15/275,043.
Inventors: Berek, Fantl. Gonzalez, Nolan, Samusik.

Invention disclosure:
Title: **A method to identify the most beneficial poly- ADP-ribose-polymerase inhibitor for cancer patients**
Submitted: 03/15/16 by Fantl, Wendy
About the Invention

Purpose:
To develop a blood test for cancer patients that will guide the selection of a PARPi maximizing efficacy while minimizing unwanted side-effects.

F. OTHER ACHIEVEMENTS

OC ambassadorship duties

Numerous seminars as above to national and international audiences.
Wendy Fantl appointed to UCSF Center for BRCA Research.

Interaction with the OC community

Movie about the work to the Stanford Women's Cancer Centre (WFantl/JBerek)
Participation in GCIG and COGi conferences in Tokyo November 2015 (WF/JB).

Your mentoring progress

Continue to mentor graduate students and post-docs in my lab (about 25) and at other institutions (GPN).
Committee member for six thesis committees (GPN).
Student mentor for three graduate students and four post-docs at Stanford (WF). One of the graduate students now applying technology he developed to our OC studies. Three post-docs are working on OC under guidance of WF and also clinical input from JB.
Graduate student mentor and pre-major advisor for eight Stanford undergraduates with interest in natural sciences and medicine (WF).
Ellen Weaver mentorship award to WF from the Bay Area Women in Science organization. Acceptance speech was focused around WF's work in ovarian cancer.

Your mentee's progress

During the past year, four of my post-docs received faculty positions (GPN).
Three graduate students graduated (GPN).
Mentoring graduate student working on ovarian cancer as one part of thesis (WF).
One undergraduate did a summer internship with WF focused on ovarian cancer. She continues to work in our lab during her school year demonstrating excellent planning and enthusiasm for the OC program.

G. CONCLUSIONS

- See tumor diversity between samples, but within *a limited phenotypic hierarchy:*
  – For both surface markers and signaling molecules
- See mutually exclusive expression of E-cadherin and vimentin in "epithelial" and "mesenchymal" compartments
- See cells in transitional EMT that also co-express stem cell markers confirming what has been proposed in the literature.
- Stem cell markers scattered throughout compartments: are there many ways to be a stem cell? Functional analysis can help answer this.
- Great diversity in size of immune compartment across samples
- We reproduce correlations between a tumor cell type with immune compartment size and immune cell type with tumor compartment size.
- We reproduced the finding that the relative size of tumor and immune compartments anti-correlated.
- We reproduce the finding that NK cell subsets are positively correlated with tumor compartment size and increase the likelihood that those NK cells could be decidual-like.
- Highly regulated communication between immune and tumor compartments.
- New level of detail regarding the immune compartment revealed by multi-parametric single cell mass

cytometry.

- We reproduced many aspects of the data from the two pilot studies which strongly validates our experiments at the technical level allowing us to makes inferences about HG-SOC biology.
- The information is foundational for following up with larger sample cohorts with the potential to inform treatments particularly immune-therapies for HG-SOC.

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